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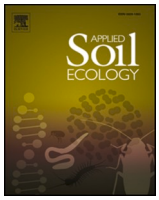

Pythium oligandrum induces growth promotion in starch potato without significantly altering the rhizosphere microbiome

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ABSTRACT

Plant health promoting organisms, including microbial biological control agents, are of increasing importance for the development of more sustainable agriculture. To understand the function of these microbes as biological control agents under field conditions and their overall impact on soil and plant health, we need to learn more about the impact of plant beneficial microbes on the rhizosphere microbiome of crops such as potato. The plant beneficial oomycete *Pythium oligandrum* has previously been reported both as a biocontrol agent and as a plant growth promoter, or biostimulant, in several crop species. To investigate the potential of *P. oligandrum* as a biostimulant in potato, we performed a series of controlled-environment bioassays in three cultivars. We showed that biostimulation of potato by *P. oligandrum* is plant genotype-specific. We confirmed the biostimulation by *P. oligandrum* in the starch potato cultivar Kuras under field conditions. We further investigated the effects of *P. oligandrum* on the potato rhizosphere microbiome, sampling individual potato plants at three time points over the growing season (representing the vegetative growth phase, flowering, and the onset of senescence). Metabarcoding using ITS and 16S amplicon sequencing revealed no significant overall effect of *P. oligandrum* application on the bacterial and fungal rhizosphere communities. However, some genera were significantly differentially abundant after *P. oligandrum* application, including some classified as plant-beneficial microbes. We conclude that *P. oligandrum* has a cultivar-dependent growth-promoting effect in potato and only minor effects on the rhizosphere microbiome.

1. Introduction

Modern agricultural management practices, such as tillage, and the input of agro-chemicals such as synthetic fertilizers, herbicides, and pesticides (Bano et al., 2021; Goffart et al., 2022; Hillocks and Cooper, 2012; Kessel et al., 2018), have increased yields over the past few decades (Deguines et al., 2014). However, extensive and continuous application of synthetic fungicides and fertilizers poses a threat to both human health and the environment (Hashemi et al., 2022). In addition, modern agriculture has led to severe declines in the biodiversity of agroecosystems (Raven and Wagner, 2021) and has negatively impacted the physicochemical properties of agricultural soils, resulting in significant loss of soil biodiversity and reductions in both soil fertility and overall health (Hartman et al., 2018; Tsiafouli et al., 2015). Thus, there is a concerted research and policy effort to reduce the use of synthetic chemicals in production systems, and instead replace them with alternatives that are less harmful and more sustainable. Biological control, or biocontrol, defined here as the exploitation of living agents to combat organisms such as pests, pathogens, and weeds for the protection of our crop, or other production, systems (Stenberg et al., 2021). It is therefore emerging as an important pillar of integrated pest management (IPM) systems which seek to reduce our reliance on synthetic inputs. Biocontrol agents may also induce biostimulation. Growth promotion is a specific form of biostimulation of plants and can be defined as a mechanism by which external stimuli improve the quality (often defined in terms of yield, or marketable value) of a crop through improving the capacity of the plant to assimilate, translocate, and use nutrients (Calvo et al., 2014). Microbes that form symbiotic relationships with plants, or

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those that live in communities closely associated with plants, such as those in close proximity to plant roots, often display both biocontrol and biostimulation properties. Thus, such organisms can be harnessed for pest and disease management, as well as growth promotion, meaning that the use of both synthetic pesticides and fertilizers can potentially be reduced in our agricultural systems.

The soil microbiome is a key determinant of soil fertility, and consequently affects plant health and productivity (Souza et al., 2015). It is defined as the community of microbes within the soil environment and includes archaea, bacteria and eukaryotic microbes. The narrow soil zone near plant root surfaces, defined as the rhizosphere, is the area where most of the plant-microbe crosstalk and interactions take place (Berendsen et al., 2012; Huang et al., 2014). Organic compounds released through root exudation attract heterotrophic microbes, including plant symbionts (Philippot et al., 2013), which in return benefit the plant, by improving nutrient uptake, growth and pathogen resistance. The plant root system, and the associated microbiomes found on and in plant roots have been likened to the human gut, since plant roots also gather nutrients and expel waste. Like their mammalian counterparts, the microbial communities the roots host play important roles in the health of the entire organism (Ramírez-Puebla et al., 2013). Thus, understanding the structure and function of rhizosphere and root-associated microbiomes has been ascribed as an important step for improving soil health and fertility and thereby plant vigour and ultimately yield (Berendsen et al., 2012).

Several attempts to manipulate the root-associated microbiome, and consequently improve plant productivity or disease resistance have been made. One emerging strategy is to add plant-beneficial microbes, singly or as mixed communities, to the soil with the intention of improving the microbiome, and thus improve traits such as overall plant health, plant protection, vigour and yield whilst lowering the input of synthetic fertilizers or plant protection products (Köhl et al., 2019; Noman et al., 2021). However, the microbiome may also be unintentionally altered when crops are sprayed with beneficial plant microbes, including microbial biocontrol agents (BCAs), or those used for biostimulation. Since plant and soil microbiomes form complex communities, which we still do not fully understand, there has been little research into the effects of altering microbiomes by the addition of microbial BCAs, although this knowledge will become increasingly important as we increase the large-scale use of BCAs in plant protection strategies.

The oomycete *P. oligandrum* is a mycoparasite of several plant pathogens (Gerbore et al., 2014; Liang et al., 2020) and a BCA with potential to control a number of plant diseases (Bělonožníková et al., 2022). In addition, it has been observed that *P. oligandrum* can promote growth in several vegetable and crop species e.g. in field grown sugar beets (Veselý, 1989), cucumbers (Kratka et al., 1994), rice plants (Cother and Gilbert, 1993), pepper plants (Al-Rawahi and Hancock, 1998), and tomatoes (Le Floch et al., 2003). Plant growth promotion by *P. oligandrum* has been associated with an increased yield in tomato plants (Le Floch et al., 2003), and overall higher plant fitness (Bělonožníková et al., 2022).

Potato (*Solanum tuberosum* L.) is one of the crops that has strongly benefitted from the intensification of agricultural systems, where yield potential has increased by almost 190 % since the 60s (Meno et al., 2021). Previous studies on potato rhizosphere microbiomes have focused mainly on bacterial and fungal spatio-temporal dynamics (Lukow et al., 2000; Zimudzi et al., 2018) between different plant growth stages (Hou et al., 2020), genotypes (Gschwendtner et al., 2011; İnceoğlu et al., 2012; Loit et al., 2020), soil types (İnceoğlu et al., 2012) and cropping systems (Larkin and Honeycutt, 2006). In this study our overall aim was to investigate if *P. oligandrum* can trigger growth promotion in potato and if amendment of *P. oligandrum* impacts the potato rhizosphere microbiome. Given the biostimulation effects seen in other plants, as outlined above, we formulated three interconnected research questions (i) *Does biostimulation occur in potatoes after amendment of the biocontrol agent P. oligandrum?* We hypothesized that *P. oligandrum* can promote growth in potato. (ii) *Is the biostimulation effect genotype specific?* We hypothesized that if present, the biostimulation effect will be genotype specific, since genotype specific biostimulatory effects have been seen in interactions between other plants and growth-promoting microbes (Schmidt et al., 2020). (iii) *Does field-application of P. oligandrum impact the rhizosphere microbiome and if so, which specific community changes can be seen?* We hypothesized that repeated treatment of field-grown potato with *P. oligandrum* will impact the native rhizosphere microbiome. We combined greenhouse and field trials, to show that *P. oligandrum* triggers growth promotion in a genotype specific manner in potato. Contrary to our expectation, we demonstrate that application of *P. oligandrum* has a minor effect on the microbial communities in the potato rhizosphere under the conditions present in our small-scale field trial. This knowledge will contribute to identifying the impact of using beneficial plant-associated microbes, such as *P. oligandrum* as an alternative solution to conventional agrochemicals, thereby paving the way for a more sustainable production of potatoes and other crops grown in open field agriculture.

2. Materials and methods

2.1. Production of Pythium oligandrum inoculum

To produce the oospore inoculum for both greenhouse and field trials, *P. oligandrum* (CBS-strain 530.74, obtained from the fungal and yeast culture collections (the CBS database) of the Westerdijk Institute, the Netherlands) was cultivated in vitro. *P. oligandrum* was maintained on solid V8 agar plates. After five days growth at 20 °C five agar plugs were transferred into 1 l bottles of clarified V8 broth and incubated in a rotary incubator for seven days with shaking at 120 rpm at 20 °C. The oospore inoculum was harvested by macerating the liquid cultures, in a high-speed blender. The inoculum was then filtered twice through miracloth and the culture media discarded. The oospores were subsequently washed and resuspended in sterile water. The initial concentration of the oospore solution was determined using a hemocytometer and adjusted with sterile water as needed.

2.2. In- vitro plant production and cultivation

Three commercially available cultivars of potatoes were used, cv. Desirée, cv. King Edward and cv. Kuras. Cultivars Desirée and King Edward are table potato cultivars and have a long history of being used in commercial production. The cultivar Kuras is a starch potato cultivar that is currently used throughout Scandinavia. All three cultivars exhibit different growth patterns, cv. Kuras and cv. Desirée are more compact and densely growing whereas cv. King Edward tends to have more elongated shoots. The plants were first propagated using *in vitro* stem cuttings of approximately 2 cm plant in Murashige and Skoog (MS) medium. The *in vitro* plant cultures were kept in a climate chamber with 16 h of light per day and a temperature of 20 °C for 10 days, until proliferate growth of shoots and roots were apparent. The *in vitro* plants were then transferred to 1 l plastic pots containing peat soil and placed in a greenhouse chamber with 16 h of artificial daylight and 24 °C. The plants were watered every second day throughout the experiment.

2.3. Field trial study site, management and potato cultivar and planting

A small-scale field trial was conducted in the common garden of SLU Alnarp in Sweden (55°66′106.2″N 13°08′198.9″E). The soil properties were characterized by EUROfins (SE). The details can be found in Supplementary Table 1 (ST 1). The fertilizers used consisted of a 100 kg nitrogen per hectare which was added in July before any treatments or rhizosphere soil sampling was done. Weeding was done by hand. For a complete overview of the management, see Supplementary Table 2 (ST 2). Certified seed tubers of the potato starch cultivar Kuras were obtained from Lyckeby SSF. The potato tubers were planted on the 07-05-

2019, 0.3–0.4 m apart, with 0.70 m between each row and six plants per row, with a total of 10 rows, covering a 7 m^2 plot.

2.4. Treatments with P. oligandrum in the greenhouse and field

In the greenhouse experiments, a total of three treatments with *P. oligandrum* oospore inoculations were carried out. Each treatment consisted of 10 ml inoculum applied as foliar spray, using a high-pressure handheld sprayer. An additional 10 ml of *P. oligandrum* oospore solution was applied as soil drench at the root bases of the plants. A final concentration of 1.25×10^4 oospore/ml, resuspended in sterile water, was used for each treatment. In total, six plants from each variety were treated in each experiment, and six plants were used as controls, which were treated with the same volume of sterile water. The experiment was repeated independently three times.

For the small-scale field trial, plants were treated five times throughout the cultivation period either with a solution of *P. oligandrum* oospores in sterile water or with the same volume of sterile water without oospores (untreated controls). Foliar application and soil drenching were carried out as described above, with a concentration of 2.5×10^4 oospores/ml at the application rate of 300 l per hectare, giving approximately 3 l per treatment, which equates to 200 ml per plant. The volume used was larger than in the greenhouse to account for the larger plants with a larger/unrestricted root mass, spacing between plants and run-off/drift from the sprayer. This is also the concentration and volume per hectare recommended when using the commercial preparation of *P. oligandrum*. Therefore, although we used a lab strain, we decided to apply it as close as possible to the potential application rate that a farmer would use in practice. The plants that were selected for treatment and microbiome sampling were fully randomized within the plot. The first treatment was carried out on the 08-07-2019 immediately after rhizosphere soil sampling and the last treatment on the 03-09-2019 just before soil sampling. The plants were treated biweekly. A total of $n = 12$ plants were sprayed with *P. oligandrum* and n = 12 plants were used as control which were sprayed with the same volume of water. The outer two plants of each row were considered border plants and not included in the experiment.

2.5. Plant growth measurements in the greenhouse and in the field

Plant growth promotion in the greenhouse experiments, was determined by harvesting the plants in the fourth week after treatment was applied. Total plant height was determined by measuring the longest shoot from the bottom of the plant root base to the apex of the shoot. Plant biomass measurements in the greenhouse experiments consisted of fresh and dry weight of the plant shoots and roots at harvest. For the dry weight measurements, the harvested shoots and roots were dried at 60 °C for 24 h and immediately after drying was completed, the sample weight was recorded. In the field trial, the final height reported in the present study was measured (16-09-2019). Plant height from a total number n = 12 plants sprayed with *P. oligandrum* and n = 12 control plants was recorded.

2.6. Rhizosphere soil sampling

Rhizosphere soil sampling was performed three times during the cultivation period. The first samples were taken at the early growth stage (08-07-2019) where no treatment was amended before sampling, thus these samples serve as baseline samples. The second time point (06-08-2019) was at the flowering stage of the potato plants, and the third time point (03-09-2019) was at the onset of senescence. These time points will hereafter be referred to as July, August and September. A total number of $n = 6$ rhizosphere soil cores were taken at each time-point at a depth of 0.2–0.3 m as described by He et al. (2022), for both control plants and plants treated with *P. oligandrum*. The sampling strategy was carried out in order to follow the individual plants over the season.

2.7. Rhizosphere soil DNA extraction

Rhizospheric soil samples were kept on ice directly after sampling and transferred to a −80 °C freezer prior to DNA extraction After thawing at room temperature each individual biological replicate was mixed well in a plastic bag. From each sample, a subsample of 0.25 g was used for DNA extraction. The DNA extraction was performed using the Qiagen DNeasy PowerSoil Pro kit, following the manufacture's protocol.

2.8. Additional control samples

Additional control samples were acquired for methodological testing and robustness of the amplicon sequencing. Here we included two different predefined mock communities. One was from ZymoBIOMICS Microbial Community Standard (Biosite-D6310) and ZymoBIOMICS Microbial Community DNA Standard (BioSite-D6306). Both were log distributed and were used as a reference for sequencing performance and potential cross contamination exclusion.

2.9. Amplicon sequencing

The primer sets, 799F (AACMGGATTAGATACCCKG) (which is chloroplast excluding) and - 1115R (AGGGTTGCGCTCGTTG) for bacteria and FITS7 (GTGARTCATCGAATCTTTG') - ITS4 (TCCTCCGCTTATTGATATGC) based on (Ihrmark et al., 2012; White et al., 1990) for fungi were ligated to a 10-nucleotide barcode and samples amplified by PCR. The PCR reaction was carried out in a volume of 20 uL containing 15 picomole of each primer, 1.5 units of MyTaq DNA polymerase (Bioline GmbH, Luckenwalde, Germany) and 2 µl of Bio-StabII PCR Enhancer (Sigma-Aldrich Co.) along with 1-10 ng DNA using an annealing temperature of 55 °C. The DNA concentration of the target amplicons was evaluated through gel electrophoresis. Approximately 20 ng of amplicon DNA from each sample was combined, resulting in pools of up to 48 samples, each tagged with unique barcodes. These amplicon pools underwent purification using Agencourt AMPure XP beads (Beckman Coulter, Inc., IN, USA) to eliminate primer dimers and other small mispriming artifacts, followed by an additional purification step using MiniElute columns (QIAGEN GmbH, Hilden, Germany). Subsequently, approximately 100 ng of each purified amplicon pool DNA was utilized to generate Illumina libraries employing the Ovation Rapid DR Multiplex System 1–96 (NuGEN Technologies, Inc., CA, USA). The resulting Illumina libraries (Illumina, Inc., CA, USA) were combined and subjected to size selection via preparative gel electrophoresis. Sequencing was performed on an Illumina MiSeq (2x300bp) utilizing V3 Chemistry (LGC Genomics, Germany). The total length of obtained reads was roughly 60 kb per sample.

2.10. Bioinformatic data analysis of microbiome data

Both the bacterial and fungal raw reads were processed using the DADA2 pipeline (Callahan et al., 2016) in R version 4.2. Default settings through the pipeline were used for filtering and trimming. Identical sequencing reads were combined using the dereplication function. Paired-end reads were merged, chimeras were removed, the amplicon sequence variant (ASV) table was constructed, and taxonomy was assigned using the SILVA database v. 138.1 and the UNITE database (https://unite.ut.ee/) for bacterial and fungal annotation, respectively. DADA2 data outputs were combined into a phyloseq object using the phyloseq-package version 1.44 by (McMurdie and Holmes, 2013) and the Microeco-package version 0.12.1 (Liu et al., 2021). R codes and further information can be found at https://github.com/Christian-Benjamin/P.oligandrum-growth-promotion-Andersen-et-al.-Applied-soil-ecology.

2.11. Statistical analysis

R-studio (version 4.1.2 2022, RStudio, Inc) was used for statistical analysis. The package rstatix (Kassambara, 2020) was used to compute different statistical tests, which included *t*-test, Wilcoxon test, ANOVA, Kruskal-Wallis and correlation analyses. Boxplots were generated using the package ggplot2 (Wickham, 2009).

Biostimulation in the greenhouse bioassays was determined by the dependent variables i) centimeters of growth of the longest shoot along with ii) fresh and iii) dry weight of the shoots and roots as an effect of treatment. To test for effects of treatment with *P. oligandrum* a standard two-way ANOVA including both treatment and experiment as predicting factors was used. The ANOVA-models were run separately for each of the tested cultivars, to fulfill the assumptions of normal distribution of the residuals. The root fresh weight was logarithmically transformed for all genotypes to obtain a normal distribution of the data. The root dry weight was square root transformed for the genotype cv. Kuras, and logarithmically transformed for the genotypes cv. Desirée and cv. King Edward. For the plant growth promotion in the small-scale field trial a standard one-way ANOVA with treatment as a factor was used.

For the microbiome data, to test for differences in the relative abundance of bacterial and fungal phyla, across time or between *P. oligandrum* versus control treatments, a series of one-way ANOVAs were used. To test for temporal and treatment differences on the alpha-diversity index, one-way ANOVAs followed by Duncan's post-hoc test ($p < 0.05$) were conducted. PERMANOVA tests (Anderson, 2017) with 999 permutations were used to test for differences in microbiome community structure between *P. oligandrum* treated and control plants. To visualize differences between treated and control plants a Principle Coordinate Analysis on the ordinations based on the Bray-Curtis dissimilarity matrix was performed. The differential abundance test was performed using the ANCOMBC-package version 1.0.5 by (Lin and Peddada, 2020). All microbiome data was analysed and visualized using a combination of phyloseq-package version 1.44 by (McMurdie and Holmes, 2013) and the Microeco-package version 0.12.1 by (Liu et al., 2021).

3. Results

3.1. Screening of biostimulation induced by P. oligandrum treatment in three different potato genotypes

To investigate biostimulation induced by *P. oligandrum* and potential genotype differences, a series of bioassays were performed in a controlled greenhouse environment involving three different potato genotypes. The plants were either treated with *P. oligandrum* oospores in sterile water or with sterile water as untreated controls. The experiment was repeated three independent times. The genotype cv. Kuras responded to the *P. oligandrum* treatment with a significantly larger plant height ($p < 0.001$) and fresh weight of both shoots and roots ($p < 0.001$, Fig. 1). The dry weight of shoot biomass ($p < 0.05$) was also significantly higher in cv. Kuras treated with *P. oligandrum* ($p = 0.031$, S Fig. 1A). However, the dry weight of the roots did not show a significant increase in comparison to the control plants for the genotype cv. Kuras ($p = 0.093$), where the effect of the experimental round was higher ($p = 0.006$, S Fig. 1D). The genotype cv. Desirée tended to grow longer shoots

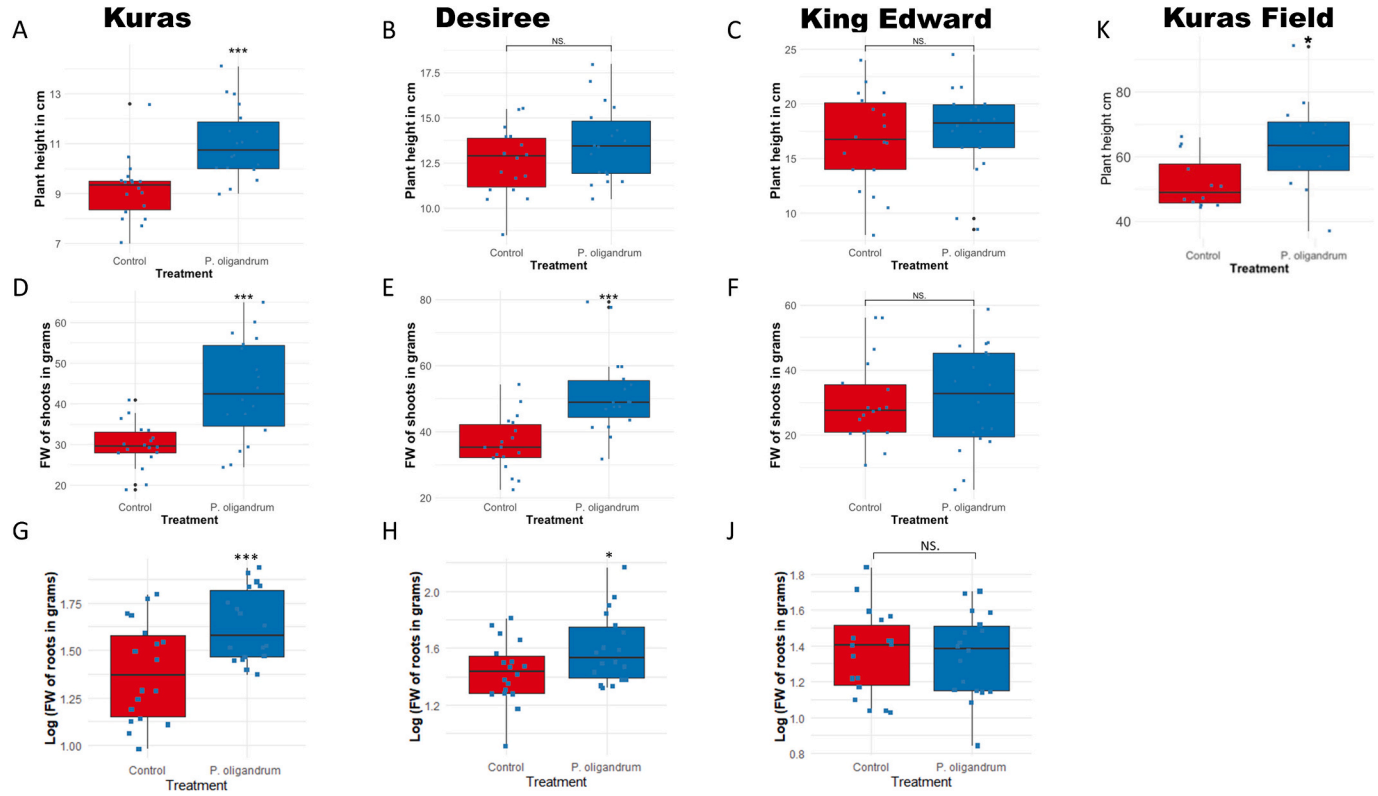

Fig. 1. Growth promotion of potato by *Pythium oligandrum.*
Biostimulation of three different potato genotypes treated with *P. oligandrum.* (A-J) Boxplots of biostimulation of potato plants treated with *P. oligandrum* (blue boxplots) or untreated controls (red boxplots), in the three different genotypes cvs. Kuras, Desirée, King Edward, in greenhouse bioassays. (A-C) Plant height of the longest shoot in cm. (D-F) Fresh weight of the shoots in grams. (G-J) Fresh weight of roots in logarithmic transformed weight in grams. NS $p > 0.05$, * $p < 0.05$,*** $p < 0.001$ The total number of plants treated, or controls were $n = 6$ per experiment per genotype. The experiment was repeated 3 times independently. (K) Boxplot of the cv. Kuras potato plant height of the longest shoot at the end of the cropping season in a small-scale field trial. The red boxplot represents untreated control plants, and the blue boxplot *P. oligandrum* treated plants. Significant difference ($p < 0.05$) is indicated with an asterisk. The total number of plants used per treatment in the field trial was $n = 12$.

as a result of the treatment, however it was not significant ($p = 0.13$, Fig. 1B). However, the fresh weight of the shoots and the roots was significantly higher after *P. oligandrum* treatment (Fig. 1E, H) for cv. Desirée with $p < 0.001$ and $p < 0.05$, respectively. The dry weight of the shoots was also significantly higher ($p < 0.05$), but not the dry weight of the roots ($p = 0.14$) (S Fig. 1B, E) in this cultivar. We did not observe any significant effect of *P. oligandrum* treatment compared to control plants in the genotype cv. King Edward (Fig. 1C, F, J and S Fig. 1C, F).

3.2. Field-grown starch potatoes can be biostimulated by P. oligandrum

Based on the positive effect on the genotype cv. Kuras in the greenhouse in response to *P. oligandrum* treatment (Fig. 1K), it was decided to use this genotype in a small-scale field trial. In the field trial the biweekly treatment with *P. oligandrum* from early July resulted in significant increase in plant height at the end of the cropping season in September (Fig. 1K), suggesting biostimulation.

3.3. Analysis of the potato rhizosphere microbiome after treatment with P. oligandrum

To investigate the impact of *P. oligandrum* treatment on the rhizosphere microbiome as well as changes over the cropping season, comparisons of the bacterial and fungal communities were made between samples collected in early July, just before the start of *P. oligandrum* treatments, with samples collected in August, at the flowering stage and in September during the onset of senescence, following the biweekly treatments of cv Kuras with *P. oligandrum*. To ensure that the results were comparable between individual samples, we first checked that there were no significant differences between the observed ASVs, or alpha or beta diversity measures between individually untreated plants at the start of the experiment (S Fig. 2A-E).

3.3.1. Analysis of the potato rhizosphere microbiome throughout the cropping season

We next investigated whether there were changes in the potato rhizosphere microbiome throughout the cropping season, by investigating changes in diversity measures over the timeframe of the experiment in either the control plants or those treated with *P. oligandrum*.

The mean relative abundance of the top 10 phyla of both bacterial and fungal phyla for both control plants and plants treated with *P. oligandrum* is seen in (Fig. 2). Detailed analysis using a one-way ANOVA comparison in the control plants revealed that the relative abundance of Actinomycetota (formerly known as Actinobacteriota) was the only phylum among the top 10 most abundant bacterial phyla that showed significant differences (S Fig. 3A). Given the constrains of predetermined phylum names in our analysis software we continue to refer to this phylum as Actinobacteriota. The phylum exhibited a decreasing trend over time, from 62.32 % abundance in July to 50.97 % in August and 50.01 % in September ($p < 0.05$). Further analysis indicated that the relative abundance in July was significantly different from August and September ($p < 0.05$), while no significant difference was observed between August and September (S Fig. 3A). In *P. oligandrum* treated plants, the relative abundance of Actinobacteriota was not significantly different among any timepoints. However, the relative abundance of Bacteroidota displayed a significant change over time (S Fig. 3B). The relative abundance significantly increased at the August time point with an increase of 6.59 % from July and decreased to 4.24 % in September, ($p < 0.05$). No significant change of the phylum Bacteroidota was found between July and September, (S Fig. 3B). In addition, the phylum Verrucomicrobiota displayed changes within the cropping season. A significant decrease in relative abundance was found between the July timepoint and the September timepoint, where July had a mean abundance of (0.35 %) and the September timepoint had a mean relative abundance of (0.13 %). No significant change was found between July and August timepoints, (S Fig. 3B).

The detailed analysis of the fungal microbiome in the control plants revealed that the mean relative abundance of the phylum

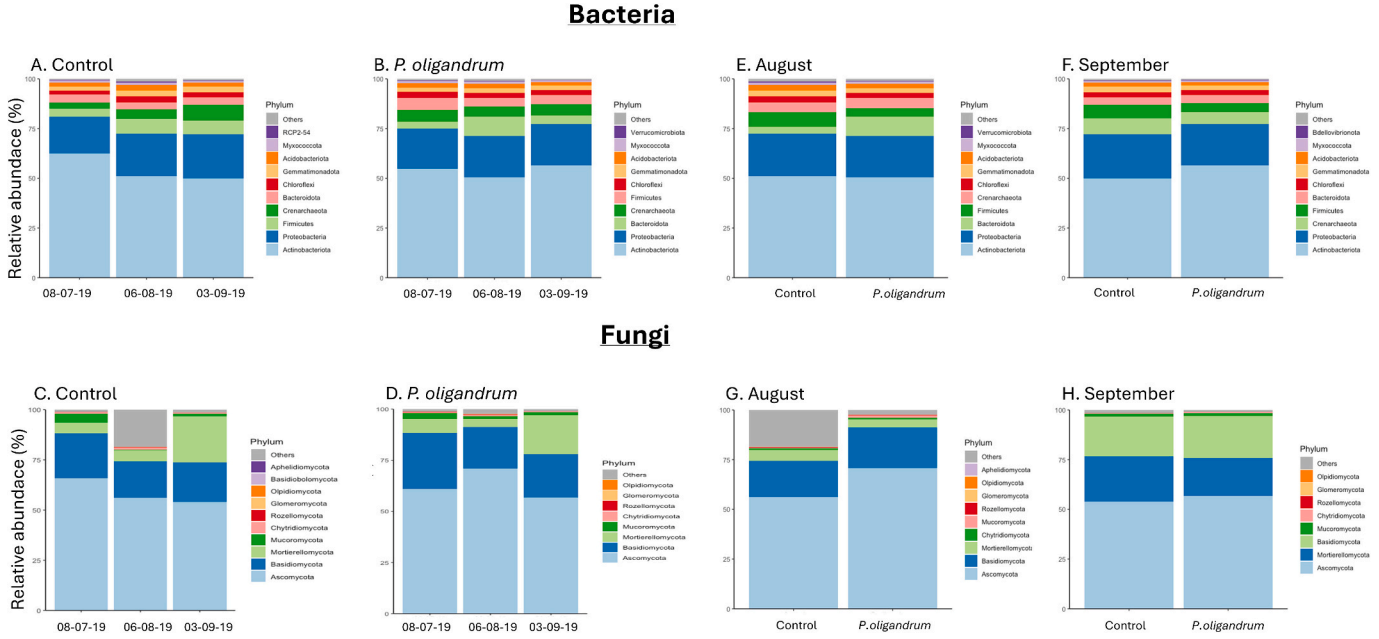

Fig. 2. The top 10 most abundant phyla in the rhizosphere microbiome of cv. Kuras potato plants treated with *Pythium oligandrum*.
The top 10 most abundant phyla in the rhizosphere microbiome of cv. Kuras potato plants in a small-scale field trial. (A) and (C) the relative abundance of bacterial and fungal phyla in the untreated control plants. (B) and (D) the relative abundance of bacterial and fungal phyla in *P. oligandrum* treated plants. The three sampling timepoints i.e. in July (08-07-19) where no treatments had been conducted, August (06-08-19) and September (03-09-19), where treatment had been conducted, are presented on the x-axis. (*E*-H) Comparison of the relative abundance of top 10 bacterial and fungal phyla between untreated control plants and *P. oligandrum* treated plants in the rhizosphere microbiome of cv. Kuras potato plants in a small-scale field trial. (E) and (G) at the August timepoint and (F) and (H) in September.

Mortierellomycota increased as time progressed (Fig. 3C). July and August had a mean relative abundance of 5.23 % and 5.43 % respectively whereas the September timepoint had a mean relative abundance of 22, 81 %. Thus, September was significantly different from July and August timepoints (S Fig. 3C). The phylum Mucoromycota displayed a significant difference in mean relative abundance between the July and August timepoint where the relative abundance decreased by 4,15 %. No significant difference was found in the mean relative abundance between July (4.53 %) and the September (1.43 %) timepoint. (S Fig. 3C).

In *P. oligandrum* treated plants the detailed analysis of the change in the mean relative abundance over the cropping season, of the top 8 fungal phyla revealed that the phylum Ascomycota was significantly increased in the August timepoint in comparison to September with an increase of 13,97 % (S Fig. 3D). No significant change was observed between August and July. The phylum Mortierellomycota had a significant increase at the September timepoint in comparison to August timepoint with an increase of 17,82 %. No significant change was observed between the August and July timepoints. Finally, we also observed that the phylum Glomeromycota had a significantly lower mean relative abundance in August in comparison to both July and September timepoints, as seen in S Fig. 3D. Relative abundance data along with statistical summaries can be found in supplemental Table 3.

Despite the minor differences observed in the relative abundance of bacterial and fungal phyla, we found no significant overall effect in alpha-diversity over time, in either the bacteria or fungal samples (Fig. 3). We further investigated the effects of sampling time within the

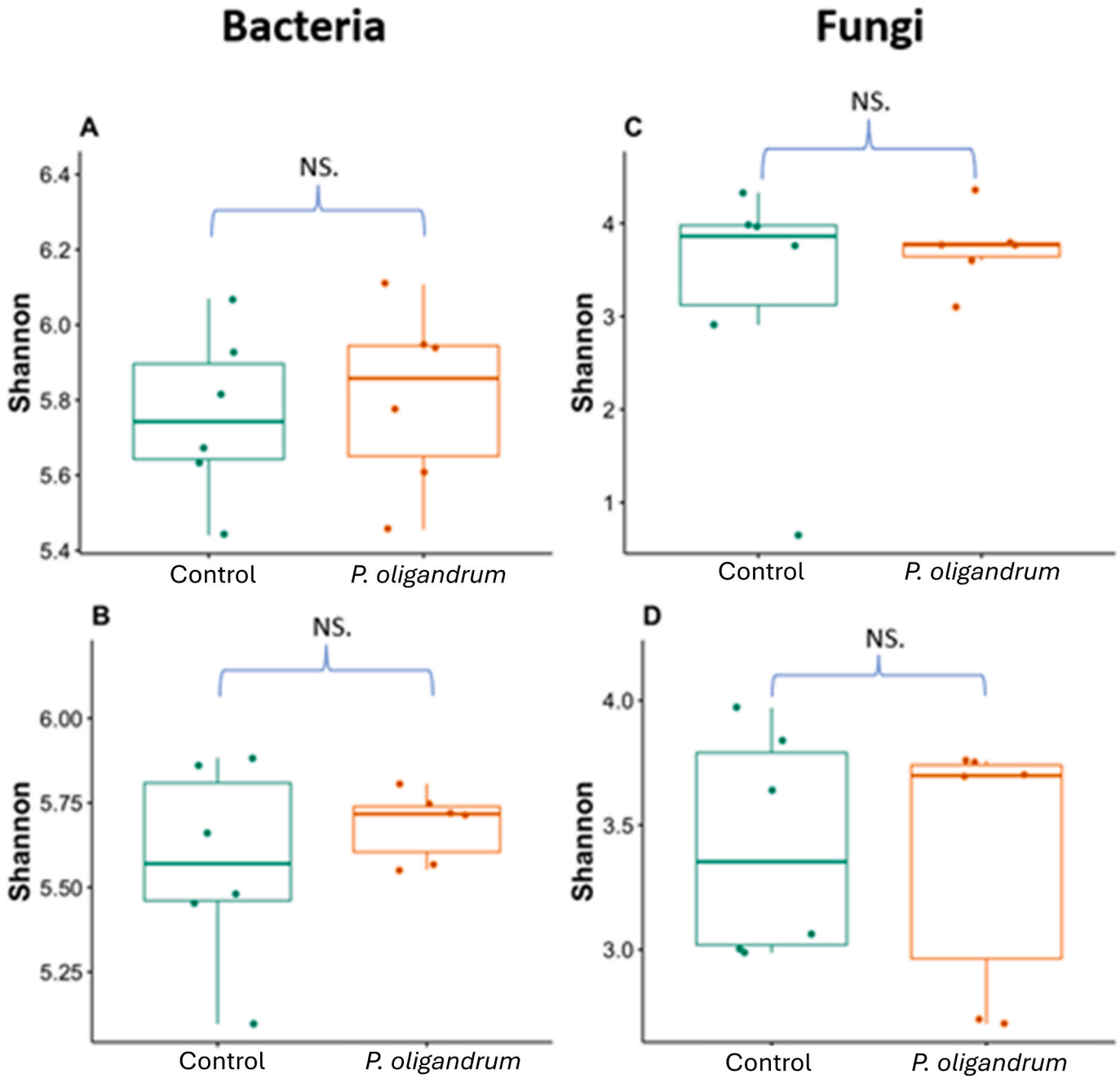

Fig. 3. Alpha diversity shown as Shannon index between untreated control plants or plants treated with *P. oligandrum* in the rhizosphere microbiome of cv. Kuras potato plants in a small-scale field trial.
(A) Bacteria samples at the August timepoint. (B) Bacteria samples at the September timepoint. (C) Fungal samples at the August timepoint. (D) Fungal samples at the September timepoint.

community structure by comparing the beta-diversity changes of the untreated plants and plants treated with *P. oligandrum* (Fig. 4). We found no significant differences in the beta-diversity of the bacterial community between time points in the control plants (Fig. 4A). The analysis of the *P. oligandrum* treated plants over the cropping season, revealed an overall significant effect on beta-diversity in the bacterial community ($p = 0.02$), see Fig. 4B. All sample timepoints of the bacteria community of *P. oligandrum* treated plants were significantly different from each other, with the strongest significant effect between July and September (Fig. 4B, $p = 0.03$). Moreover, in the fungal community we found an overall significant effect on the beta-diversity of the control plants over time (Fig. 4C, $p = 0.03$). The pair-wise comparison showed that a significant difference was found between July and September (Fig. 4C, $p = 0.012$) in control plants. No significant differences were seen between individual timepoints as a result of *P. oligandrum* treatment on the fungal community (Fig. 4D). However, the analysis showed an overall significant effect of sampling time (Fig. 4D, $p = 0.015$).

To further investigate changes during the cropping season we performed a differential abundance analysis at the genus level (S. Fig. 5). The differential abundance analysis revealed that several bacterial and fungal genera were found to be differentially abundant at the beginning of the experiment (in July) in the samples taken from the control plants. In the plants treated with *P. oligandrum* the highest number of differentially abundant bacterial genera were found at the August timepoint, whilst there were approximately equal numbers of differentially abundant fungal genera found in July and in August. Thus, whilst there are changes in differential abundance over time (as seen in the control plants) there were slightly more changes over time after treatment with *P. oligandrum* (S. Figs. 5, 6 and supplementary table ST4). Differentially abundant fungal genera that were more relatively abundant over time, in plants treated with *P. oligandrum* included 18 genera that contain various species that are plant pathogens as well as those that are considered plant beneficials (S. Figs. 5 and 6).

3.3.2. Treatment with *P. oligandrum* has a limited impact on the rhizosphere microbiome

To further understand the impact of *P. oligandrum* on the potato rhizosphere microbiome, we next compared the microbiomes of plants treated with *P. oligandrum* to the control plants to look for changes occurring as a result of treatment only, and not of sampling timepoint. Comparing *P. oligandrum* treated plants and the control plants at the two last timepoints i.e. August and September (during the time when *P. oligandrum* was applied), revealed that the application of *P. oligandrum* had a limited impact on the mean relative abundance of the top 10 bacterial and 8 fungal phyla as seen in Fig. 4.

However, the detailed analysis (using a one-way ANOVA), revealed that the mean relative abundance of the bacterial phylum Bacillota (formerly Firmicutes) was significantly lower in the plants treated with *P. oligandrum* (4.22 %) in comparison the control plants (7. 29 %) (S Fig. 4A). No significant change was seen in the mean relative abundance of the top 10 bacterial phyla at the September timepoint between plants treated with *P. oligandrum* and control plants (S Fig. 4B).

For the mean relative abundance of fungal phyla in August only the phylum Mucoromycota was significantly decreased in the control plants in comparison to the *P.* oligandrum treated plants (S Fig. 4C). No significant difference was observed in the relative abundance of the top 8 fungal phyla between control and *P. oligandrum* treated plants in September (S Fig. 4D).

There was no significant change in the overall alpha-diversity in the bacterial rhizosphere samples at either of the time points (Fig. 3A and B, respectively). A similar observation was made for the fungal rhizosphere samples, where no significant change in the Shannon index was found (Fig. 3C and D). The bacterial community structure between the *P. oligandrum* treated plants and the untreated control plants in either August or September showed a similar distribution of samples with no clear separation (Fig. 4E and F). Similarly, no clear separation due to the treatment was observed for the fungal community structure (Fig. 4G and H). The PERMANOVA test further confirmed that there were no

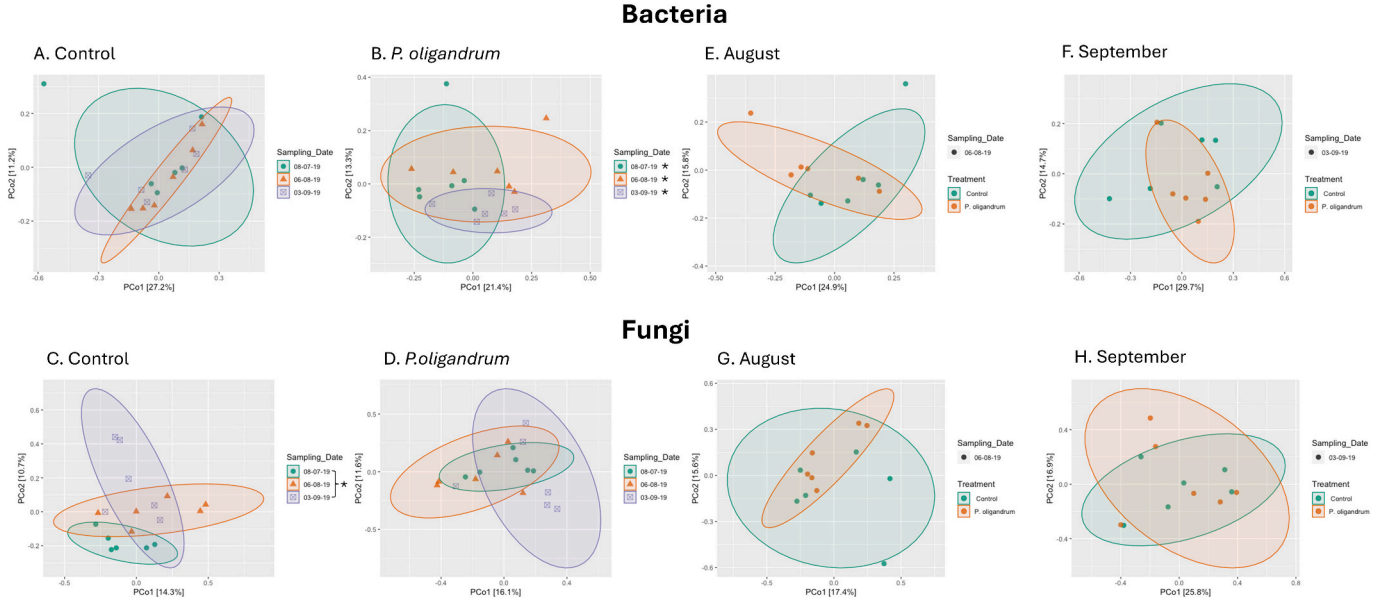

Fig. 4. Beta-diversity of change in the community structure in the rhizosphere microbiome of cv. Kuras potato plants over time and after treatment with *Pythium oligandrum*.

Panel (A-D) shows the beta-diversity of the temporal change in the community structure in the rhizosphere microbiome of cv. Kuras potato plants in a small-scale field trial. (A) and (B) bacteria and (C) and (D) fungal samples shown as PCoA plots. The sampling was done at the 3 timepoints i.e. in July (08-07-2019) where no treatments had been conducted, and in August (06-08-2019) and September (03-09-2019), where *P. oligandrum* treatment had been conducted in the field trial. (A) and (C) represents untreated control plants and (B) and (D) *P. oligandrum* treated plants. Asterisks indicate significant difference between timepoints, $p < 0.05$. (E-H) shows beta-diversity shown as PCoA plots between rhizosphere samples from either control plants or plants treated with *P. oligandrum* in the rhizosphere microbiome of cv. Kuras potato plants in a small-scale field trial. (E) Bacteria samples in August. (F) Bacteria samples in September. (G) Fungal samples in August. (H) Fungal samples in September.

significant changes in the beta-diversity of the rhizosphere community structure because of the treatment for either the bacterial or fungal community.

We next explored differential abundance at the genus level after treatment with *P. oligandrum*. Looking only at the effect of *P. oligandrum* application within each time point, (an overall analysis over time is presented in S. Fig. 5) we identified two bacterial genera with an increased relative abundance in August (*Rhidanobacter* and *Cellulosimicrobium*) (Fig. 5A). There were no differentially abundant genera in the September bacterial samples. In the fungal rhizosphere microbiome at the August timepoint, there were two differentially abundant genera (Fig. 5B). These were *Candida* and *Apiotrichum*, which were both more abundant in the *P. oligandrum*-treated rhizosphere samples. At the September timepoint of the fungal rhizosphere samples, there was one differentially abundant genus, *Coprinellus*, which was more abundant in the *P. oligandrum* treated rhizosphere samples (Fig. 5C).

4. Discussion

In this study we investigated whether the biocontrol agent *P. oligandrum* could promote biostimulation in potato. Because application of *P. oligandrum* - either to promote growth or to act as a biocontrol agent of disease - may unintentionally impact the potato microbiome, we also studied the effect of *P. oligandrum* on the rhizosphere microbiome. Results from the present study showed that *P. oligandrum* can induce growth promotion in potato both in the greenhouse and in the field, although the growth promotion seems to be genotype specific. The investigation of the potato cv. Kuras rhizosphere microbiome in a field trial showed some changes during the cropping season. These changes were mainly in beta-diversity, that is variability in the community composition between samples, whereas alpha diversity (species richness and evenness) was not significantly different over time. Moreover, although there were a few differences in the abundance of some minor genera in the microbiome after treatment

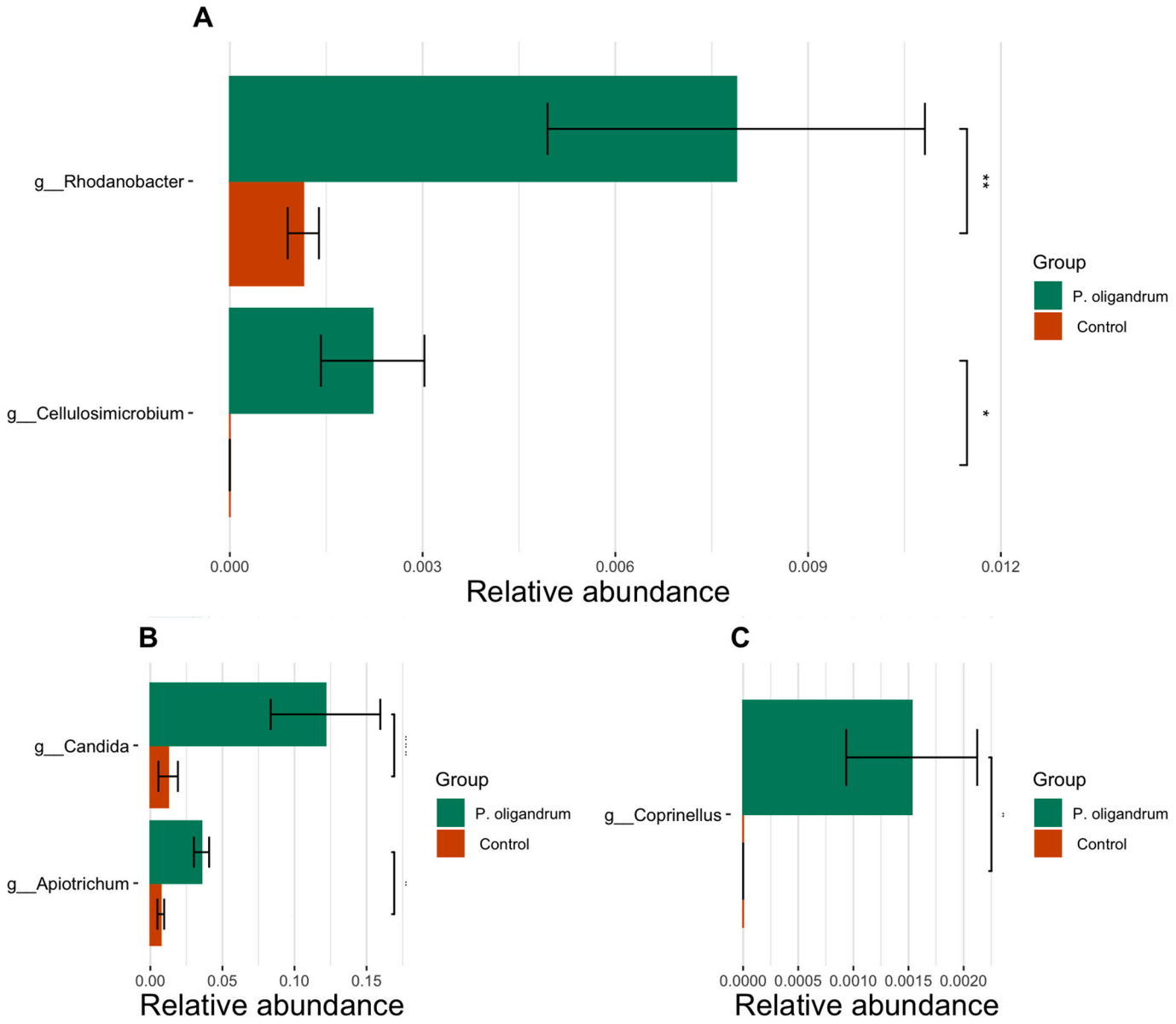

Fig. 5. Differential abundance analysis of genera, between rhizosphere samples of control plants and plants treated with *Pythium oligandrum* in the rhizosphere microbiome of cv. Kuras potato plants in a small-scale field trial.
(A) Differentially abundant bacterial genera in August (B) Differentially abundant fungal genera in August (C) Differentially abundant fungal genera in September. Asterisks indicate significant differences ($p < 0.05$).

with *P. oligandrum*, we found no evidence for major changes in the rhizosphere microbiome in potato treated with *P. oligandrum* in this small-scale field trial.

4.1. *P. oligandrum increases plant height and fresh weight biomass in the potato cultivar Kuras*

Our study revealed a significant increase in the shoot length of the starch potato cultivar Kuras when *P. oligandrum* was applied in both greenhouse and field experiments. The greenhouse study suggested that the increased shoot length, along with increased shoot and root biomass, was genotype specific. A similar genotype response to biological control agents has been reported for sugar beets treated with the biocontrol agent *Trichoderma* ssp. (Schmidt et al., 2020). For a biocontrol agent to function well their positive effects should be as consistent as possible. Therefore, the detected genotype effect in the growth promoting ability by *P. oligandrum* could decrease the applicability of this organism in agriculture where several potato varieties are used. However, the biocontrol effect of *P. oligandrum* should still be of value in non-responsive genotypes, assuming of course that there is not a variation in biocontrol capability based on interactions with specific plant genotypes as well. The fact that there is a genotype effect also means that this could possibly be exploited in the future, for example by identifying genes and markers for hosting biocontrol agents that could be used in breeding programs. The use of cultivars developed in this way could aid in the development of biocontrol solutions that function more robustly in field crops such as potato.

The increased plant height, observed after treatment with *P. oligandrum* might be caused by an increased tryptamine availability since several studies indicate that *P. oligandrum* increases the availability of tryptamine a precursor of indole-3-acetic acid (Bělonožníková et al., 2022; Le Floch et al., 2003) in plant hosts, or also in secretomes (Bělonožníková et al., 2020). Another study proposed that plant growth induced by *P. oligandrum* could be the result of an increased phosphorous uptake (Kratka et al., 1994). It was beyond the scope of the present study to elucidate the mechanism involved in increased potato plant growth. In future research, it would be of interest to fully elucidate the genotype response of *P. oligandrum* on potatoes and its mechanism.

Based on the greenhouse studies we conducted a small-scale field trial with the genotype cv. Kuras since it responded positively in biostimulation due to the *P. oligandrum* treatment. We clearly showed that repeated *P. oligandrum* application resulted in increased growth, measured as plant height, of cv. Kuras at the end of the growing season under field conditions. A study of 103 commercial potato cultivars, including cv. Kuras, suggested that plant height at the end of the growing season is positively correlated with both canopy cover and tuber weight (Aliche et al., 2018). Thus, although we were not able to measure tuber yield in the current pilot study, these data suggest that an increase in plant height could also correspond to an increase in yield in this cultivar after treatment with *P. oligandrum*. The increased shoot length (plant height) and increased biomass of cv. Kuras observed in the present greenhouse study indeed indicates that the treatment with the biocontrol agent increases plant vigour. An increased biomass has also been reported in other Solanaceous crops (Habib et al., 2021). Le Floch et al. (2003) reported an increased tomato yield due to *P. oligandrum* treatment, via production of the auxin precursor, tryptamine. *P. oligandrum* also triggers plant immunity, through secretion of two glycoproteins that have elicitin activity (Masunaka et al., 2010), activates plant defence responses (Benhamou et al., 2012) and can moderate iron homeostasis in plant roots (Cheng et al., 2022). It would be important to study if these positive effects also can make the plant more prone to combat diseases and reduce stress induced yield losses and/or if these results will lead to an increase in overall yield.

4.2. *Changes in the potato rhizosphere microbiome over time*

Plants were followed throughout the growth season and the rhizosphere microbiome was assessed for changes in alpha-diversity (Shannon, 1948) and beta-diversity (Whittaker, 1972) of bacterial and fungal species throughout the season. The analysis showed that temporal effects seem to have little to no impact on the alpha and beta diversity of the bacteria rhizosphere microbiome of untreated plants. This is somewhat contradictory to the observations made by Hou et al. (2020), who, in a larger study, analysed rhizosphere samples of potato crops in China and found a strong correlation between the growth stage of potato plants and the diversity and community structure of both bacteria and fungi in the microbiome. Our study was a pilot study conducted at a very small-scale and thus differences that may have been apparent in a larger-scale field trial may have been missed. Furthermore, we utilized a non-destructive sampling method, where rather than removing whole plants for processing, we took soil cores containing roots, from which we then separated out rhizosphere soil. This means that we may have sampled a smaller portion of the rhizosphere than in other studies in which the whole plant was removed from the ground for processing.

İnceoğlu et al. (2012) demonstrated that potato plants selectively recruited bacteria in a genotype specific manner but only at the very early growth stage and overall it remained unclear to what extent cultivar type and/or growth stage affect the bacteria microbiome assembly of field grown potato crops. Our study included only a few time points and no analysis of very young plants; thus, it is likely that we did not catch these early changes in the microbiome. In treated plants we did not see a statistically significant change in the alpha-diversity from the sampling before first *P. oligandrum* application to the end of the cropping season. Untreated control plants showed an overall significant change of the fungal community structure over time in our study. This temporal change of the fungal rhizosphere community in the potato microbiome was in line with Zimudzi et al. (2018), who observed that alpha diversity of the rhizosphere fungal microbiome was stable within the growing season, but beta diversity differed between growth stages of the plants. Thus, our observations are comparable to other studies.

Our data also suggest that *P. oligandrum* induced changes at the genus level in the potato rhizosphere microbiome over the cropping season, as seen in the differential abundance analysis. Some of the most abundant genera at the later timepoint included the fungal genus *Mortierella*. *Mortierellamycota* are associated with potato crops (Semenov et al., 2022), and have been suggested to be core fungal genera of potato plants (Imam et al., 2021). Other identified genera were *Pseudopithomyces*, *Paramyrothecium*, *Fusarium*, *Humicola and Coprinellus*, all including both plant pathogens, plant symbionts and plant growth promoting bacterial genera. Examples of the latter are for instance *Kitasatospora* and *Methylorosula*. Thus, it would be interesting to follow these observations up in future studies, for example, testing the specific interactions of these genera on potato physiology and growth as well as following up on their interactions with *P. oligandrum* in the potato rhizosphere.

4.3. *Treatment with P. oligandrum has a minor effect on the microbiome*

Although Hashemi et al. (2023) recently assessed the impact of *P. oligandrum* application on the rhizosphere microbiome of the legume *Medigago truncatula*, this study was conducted as a pot trial in controlled conditions and thus we are still lacking knowledge on the impact of this organism on the microbiome of field grown plants. Therefore, we assessed the impact of *P. oligandrum* application on the potato rhizosphere microbiome under field conditions. The relative abundance of the top 10 phyla between control plants and *P. oligandrum* treated plants were very similar and were dominated largely by the same phyla for both August and September timepoints. We found no significant impact on alpha or beta diversity after foliar and soil-drench application of *P. oligandrum* in the field-grown potatoes, at either of the two timepoints sampled. *P. oligandrum* also had no significant impact on alpha diversity

of the *M. truncatula* rhizosphere microbiome (Hashemi et al., 2023), however beta diversity changes were observed. It should be noted that Hashemi et al. (2023) studied the effects of *P. oligandrum* in container-grown plants under controlled conditions and thus differences may have been amplified within the confines of the pot experiment, compared to our field-based study.

Our results are in line with a recent study where a mix of *Bacillus subtilis* QST 713 and *Trichoderma* sp. TW2 on tomato plants had no significant impact on the resident microbiome (Cucu et al., 2020). However, other studies have found significant changes in the rhizosphere microbiome when adding BCAs. For example, Huang et al. (2021) took a similar approach to Cucu et al. (2020) using *B. subtilis* and *Trichoderma harzianum* and found alterations in the resident rhizosphere microbiome in ginger, in a dose-dependent manner. Another study by Stummer et al. (2022) used two *Trichoderma* strains against crown rot and found a significant impact on the fungal resident rhizosphere microbiome of wheat. A combination of *Bacillus* BCA strains and *Trichoderma* have also been shown to have a significant impact on alpha and beta diversity of potato crops (Wang et al., 2019). This observation is different from the study by Imam et al. (2021) who found that treatment with *Bacillus amyloliquefaciens* had a significant effect on the bacteria community structure, but nonetheless the genotype effect was a stronger predictor of the changes found in beta-diversity. In Imam et al. (2021) the application of *B. amyloliquefaciens* also showed an impact on the rhizosphere microbiome of potato plants, although this impact might be explained by the secretion of antimicrobial molecules with a broad host range by this bacterium. In contrast, the secretion of antibiotics by *P. oligandrum* is not well documented. Studies have so far shown that one of the main mechanisms of mycoparasitism exhibited by *P. oligandrum* is secretion of carbohydrate-active enzymes to breach fungal or oomycete cell walls (Liang et al., 2020) as well as mycoparasitism by coiling around prey (Gerbore et al., 2014). Thus the secretion of antibiotics that can kill microbes without close contact with the prey (antibiosis) may not be a significant mode of action of this oomycete in the same way that it is in bacteria such as *B. amyloliquefaciens*.

It is very difficult to predict whether small changes in the composition of the microbiome will have a significant impact on soil or plant health. A recent study in tomato suggests that minor changes in the composition of an individual plant's early life microbiome can cascade into differential health outcomes later in life (even when alpha or beta diversity measures are not significantly different) (Wei et al., 2019). It is also interesting to note that plants secrete molecules to attract and actively recruit beneficial microbes, which may for example be important for health outcomes of older plants. Recently it has been hypothesized that beneficial plant-associated microbes secrete effectors, or other molecules that act in concert with these plant-associated molecules to aid in their own establishment in the plant holobiont (Snelders et al., 2022). Thus, the changes in relative abundance of individual genera or species seen in potato after treatment with *P. oligandrum* could indicate this oomycete is manipulating the rhizosphere microbiome as hypothesized by Snelders et al. (2022). For example, attractants secreted by *P. oligandrum* that work together with plant attractants, might also attract mycorrhizal partners such as *Claroideoglomus claroideum.* This genus showed a higher differential abundance in rhizosphere samples inoculated with *P. oligandrum* in our study. Interestingly, Hashemi et al. (2023) found that *P. oligandrum* did not impair colonisation of *M. truncatula* roots by AMF fungi and increased subsequent root colonisation by nitrogen-fixing bacteria. *P. oligandrum* is a mycoparasite that predates a large number of fungi and other microbes (Bělonožníková et al., 2022), and thus we might expect changes in the fungal community due to predation within the rhizosphere. It has also recently been suggested that this mycoparasite has a preference for fungal prey from the Ascomycota rather than the Basidiomycota (Hashemi et al., 2023), which may also explain why we see some of the specific minor changes in the rhizosphere microbiome in our study. Many of the minor changes in differential abundance seen in our study were of fungal genera which include plant beneficial species. Hashemi et al. (2023) also noted that *P. oligandrum* may favour the recruitment of similar plant beneficial microbes to the plant rhizosphere. It would therefore be of interest to further investigate the connection between specific changes in the rhizosphere microbiome with the observed plant growth promotion induced by *P. oligandrum* treatment in the future, to identify if *P. oligandrum* is actively recruiting specific beneficial species, or repelling competitors. Future research should also investigate the potential of deliberately manipulating or engineering the rhizosphere microbial community of potato plants to better support overall plant health and crop yield.

Our study was conducted on a very small-scale with a limited number of replicates and thus we cannot be sure that our results are simply a result of the experimental set up and not fully representative of the data that would be obtained in a larger, commercial field. Although we did not check for viability of *P. oligandrum* throughout our experiment, we repeatedly applied fresh inoculum throughout the trial to ensure that we maintained a population of living *P. oligandrum* within the system. Using the same method, we were able to show a biocontrol effect in a parallel study (Stridh et al., 2022). Furthermore, in a previous study we specifically investigated the presence of native *P. oligandrum* strains in the same field site (Vetukuri et al., 2020). However, we could not detect any members of this species in that study (Vetukuri et al., 2020), and thus we believe that the effects we saw in the current study are due to our repeated treatments with *P. oligandrum.*

Although we tried to choose the best set of experimental conditions possible, including the use of mock communities and the choice of primers for metabarcoding, our experimental set up may also have introduced bias into the results. A slightly different picture may have been obtained if we had used different sets of ITS primers, since they are well known to exhibit bias (Tedersoo et al., 2022), different sequencing platforms and/or alternative bioinformatics pipelines for example including a database geared entirely towards the soil microbiome only, since these too can exert bias on the analysis outcomes (Pauvert et al., 2019). This could also be the case if we had sequenced root microbial communities and/or additional rhizosphere samples per plant. It might also be that the rhizosphere microbiome of potato cultivar Kuras, grown in our conditions, is not easily manipulated and requires larger inputs to destabilize than the genotypes used in other published studies. Alterations in resident microbiomes due to BCA applications are likely dependent on several other interlinked factors such as crop species/genotype, study system, soil, and climate factors as well as microorganism used, making it difficult to draw too many comparisons between different data sets.

5. Conclusions

In conclusion, this study demonstrates *P. oligandrum* promotes biostimulation, in a genotype-dependent manner in potato. This suggests that the genetic background of potato plants impacts the benefit of using this biocontrol agent. It may also imply that it plays a role in the recruitment of plant beneficial microorganisms, and future research should investigate this in more detail. The cv. Kuras microbiome undergoes changes during the cropping season, with and without *P. oligandrum* application. The treatment with *P. oligandrum* did not impact the overall alpha and beta diversity of the bacterial and fungal microbiome within the present study. However, treatment with *P. oligandrum* did result in some genera being differently abundant in comparison to the untreated control plants, and future research is needed to unravel their role in the biostimulation effects observed in this study. Collectively *P. oligandrum* should be regarded as a biostimulant of some genotypes of potatoes, which may only have minor effects on the potato rhizosphere microbiome, although more research in larger scale trials is needed to confirm this.

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CRediT authorship contribution statement

Christian B. Andersen: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kristin Aleklett:** Writing – review & editing, Validation, Methodology, Formal analysis. **Garima Digdarshika:** Writing – review & editing, Formal analysis. **Åsa Lankinen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Laura J. Grenville-Briggs:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data (raw & analysed & supplemental) can be found in the following Github repository: https://github.com/Christian-Benjamin/P.oligandrum-growth-promotion-Andersen-et-al.-Applied-soil-ecology

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